

AD _____

Award Number: DAMD17-00-1-0180

TITLE: Administration of Additional Phosphorylated Prolactin
During Pregnancy Inhibits Mammary Ductal Branching and
Promotes Premature Lobuloalveolus Development

PRINCIPAL INVESTIGATOR: Ameae M. Walker, Ph.D.

CONTRACTING ORGANIZATION: University of California
Riverside, California 92521

REPORT DATE: July 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20011128 057

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 074-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503				
1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE July 2001	3. REPORT TYPE AND DATES COVERED Annual (19 Jun 00 - 18 Jun 01)		
4. TITLE AND SUBTITLE Administration of Additional Phosphorylated Prolactin During Pregnancy Inhibits Mammary Ductal Branching and Promotes Premature Lobuloalveolus Development		5. FUNDING NUMBERS DAMD17-00-1-0180		
6. AUTHOR(S) Ameae M. Walker, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of California Riverside, California 92521 E-Mail: Ameae.Walker@ucr.edu		8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSORING / MONITORING AGENCY REPORT NUMBER		
11. SUPPLEMENTARY NOTES Report contains color photos.				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 Words) Prolactin (PRL) is a hormone recognized as having both proliferative and differentiative activities in the mammary gland. Current theory proposes that it is the coexisting steroidal environment which dictates whether PRL is proliferative or differentiative. Preliminary data, presented as part of the initial proposal, however, suggested that it was the form of PRL released that dictated whether proliferation or differentiation would occur. By form of PRL we mean whether the PRL is released from the pituitary as an unmodified polypeptide or whether it is phosphorylated. To study this issue, we have produced recombinant, unmodified PRL (U-PRL) and a recombinant, molecular mimic of phosphorylated PRL, S179D PRL. In the studies conducted during the first year, we have confirmed our initial observations that U-PRL promotes growth, while S179D PRL inhibits growth and promotes differentiation. These effects were shown to be independent of progesterone, estrogen, corticosterone and placental lactogens and hence are likely to be direct effects of the different PRL forms on the mammary gland. In vitro studies have shown that treatment with S179D PRL markedly increases the ratio of short to long receptors, suggesting a mechanism which could mediate a switch from a proliferative to a differentiative response.				
14. SUBJECT TERMS Mechanism of hormone action, growth factors/cytokines			15. NUMBER OF PAGES 33	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

Table of Contents

Cover.....	1.....
SF 298.....	2.....
Table of Contents.....	3.....
Introduction.....	4.....
Body.....	4.....
Key Research Accomplishments.....	5.....
Reportable Outcomes.....	5.....
Conclusions.....	5.....
References.....	6.....
Appendices.....	7-33.....

INTRODUCTION

An understanding of the hormonal influences on growth and differentiation in the mammary gland is crucial to the development of preventative strategies and additional treatments for breast cancer. Prolactin (PRL) is a hormone that has long been described as promoting both growth and differentiation in the mammary gland and it has been proposed that it is the coexisting steroidal environment that dictates which of the two activities PRL displays (1). The hypothesis underpinning this grant suggests instead that it is the different molecular forms of PRL that result in proliferation versus differentiation. In the rat, which serves as our experimental model, two main forms of PRL are produced, unmodified and phosphorylated PRL (2,3). We have made recombinant versions of these two PRLs (4) to further test this hypothesis and to also determine whether exposure to phosphorylated PRL brings about a degree of differentiation that makes the gland refractory to carcinogenesis.

BODY

In the first 12 month period, we have conducted the first in vivo experiment designed to determine whether the effects of the recombinant PRLs on the development of the pregnant mammary gland were direct or indirect. These results are described in detail in the attached manuscript, which is currently submitted for publication. Briefly, these studies confirm our original observations that unmodified PRL (U-PRL) promoted ductal and alveolar growth, while the molecular mimic of phosphorylated PRL (S179D PRL) inhibited ductal growth and branching, promoted the formation of alveoli and promoted differentiated function. Hormone assays and the conduct of additional experiments in non-pregnant animals determined that the different PRL activities were independent of progesterone, estrogen, corticosterone and placental lactogens. Funds from another source also allowed us to determine that treatment with S 179D PRL was associated with an increase in the short to long PRL receptor ratio, which was itself positively correlated with increased β -casein expression. It is likely therefore that differentiative function is mediated through this switch in receptor ratios – see attached abstracts. This part of the project is ahead of schedule with the manuscript already complete.

Also in the first 12 month period, we have begun the experiment to test whether exposure to phosphorylated PRL is the key to the refractoriness to carcinogenesis brought about by pregnancy. This experiment, utilizing NMU induction of ductal carcinomas, is ongoing and it is too early to report the results. In this regard we are a little behind schedule due predominantly to a problem during the Fall with the cells used for our bioassay. We did not want to take the risk of starting a very expensive and time-consuming animal experiment on the basis that our protein preparations were probably good. We felt it necessary to be sure and hence had to wait until we could solve the cell problem.

Pilot organ cultures have been tested to establish the technique in the laboratory and we are now in a position to begin those experiments which were planned for the 12-21 month period of the grant.

KEY RESEARCH ACCOMPLISHMENTS

Establishment

That the different forms of PRL have different roles in the mammary gland

- That unmodified PRL has a growth-promoting effect on mammary ducts which is independent of progesterone, but which likely synergizes with the effect of progesterone
- That administration of S179D PRL can inhibit the normal growth of the mammary gland during pregnancy when a large amount of ductal growth and branching occurs
- That S179D PRL promotes differentiation, as evidenced by expression of the milk protein, β -casein.
- That a switch from proliferation to differentiation is associated with an increase in the ratio of short to long PRL receptors and that this may be key to the mechanism.

REPORTABLE OUTCOMES

- 1) 1 manuscript currently submitted for publication (appended) entitled "pseudophosphorylated prolactin (S179D PRL) inhibits growth and promotes differentiation in the rat mammary gland"
- 2) 2 abstracts/ presentations made at the Annual Meeting of the Endocrine Society, Denver, CO, June 2001. The abstract entitled "signaling and biological activity of a molecular mimic of phosphorylated prolactin" was presented in a symposium. This covered work supported by several grants over the years, but the more recent work on the mammary gland was attributed to support by the USARMC. The second abstract entitled "differential modulation of the expression of the long and short form of the PRL receptor and β -casein by unmodified PRL and a molecular mimic of phosphorylated PRL suggests that the short form of the receptor does not act as a dominant negative for signaling resulting in β -casein expression" was presented as a poster.

CONCLUSIONS

The results thus far have solidified the hypothesis that phosphorylated PRL (and the mimic, S179D PRL) can cause mammary gland differentiation. S179D PRL inhibits ductal growth by promoting the premature formation of alveoli from terminal end buds (equivalent to terminal ducts in humans). It is therefore possible that exposure to phosphorylated prolactin in pregnancy is what affords refractoriness to carcinogenesis and it may be possible to utilize the S179D PRL as a treatment to duplicate this effect in women delaying their childbearing. These two possibilities are the subject of experiments to be conducted in the next two years of the grant.

REFERENCES

- 1) Vonderhaar B. Regulation of the development of the normal mammary gland by hormones and growth factors. In: Lippman M, Dickson R eds. Breast cancer: cellular and molecular biology. Kluwer Academic Publishers, Norwell, Massachusetts; 1988:252-266
- 2) Ho TW, Leong FS, Olaso CH, Walker AM. Secretion of specific non-phosphorylated and phosphorylated prolactin isoforms at different stages of the estrous cycle. *Neuroendocrinology*. 1993;58:160-165
- 3) Ho TWC, Kawaminami M, Walker AM. Secretion of phosphorylated and non-phosphorylated monomer prolactin isoforms during rat pregnancy and pseudopregnancy. *Endocrine J* 1993;1:435-439
- 4) Chen T-J, Kuo CB, Tsai KF, Liu J-W, Chen D-Y, Walker AM. Development of recombinant human prolactin receptor antagonists by molecular mimicry of the phosphorylated hormone. *Endocrinology* 1998;139:609-616

Pseudophosphorylated prolactin (S179D PRL) inhibits growth and promotes differentiation in the rat mammary gland

C. Benson Kuo, Wei Wu, Xiaolei Xu, Lili Yang, Cyndi Chen, Djurdjica Coss, Ben Birdsall, Dorsa Nasserli and Ameae M. Walker*

Division of Biomedical Sciences
University of California
Riverside, CA 92521-0121

Running Title: Phospho PRL promotes differentiation

Key Words: unmodified prolactin, phosphorylated prolactin, duct, alveolus, β casein

* To whom correspondence should be addressed

FAX: (909) 787-5504

Telephone: (909) 787-5942

Email: ameae.walker@ucr.edu

Supported by DOD Breast Cancer Research Program Grant #BC 990711 and a grant from Sensus Drug Development Corp., Austin, Texas

ABSTRACT

In the current study, we have investigated the individual roles of unmodified prolactin (U-PRL) and phosphorylated PRL in mammary development. In the first two protocols, recombinant versions of the PRLs were delivered to rats throughout pregnancy at a rate of 6 $\mu\text{g}/24\text{h}/\text{rat}$. In the second two protocols, the same PRLs were delivered to non-pregnant females at a rate of 24 $\mu\text{g}/24\text{h}/\text{rat}$. Measurement of progesterone, corticosterone and estradiol showed no effect of the administered PRLs on the levels of these other mammotropic hormones in either protocol. Histological analysis showed U-PRL to cause ductal and alveolar growth, while the recombinant molecular mimic of phosphorylated PRL, S179D PRL, inhibited ductal growth and promoted the formation of alveoli. Molecular analysis showed decreased β casein expression in the mammary glands of the U-PRL-treated animals at term and increased β casein expression in the mammary glands of the S179D PRL-treated animals. Superior β casein gene expression in response to S179D PRL versus U-PRL was confirmed in HC11 cells. We conclude that U-PRL is important for growth in the mammary gland while phosphorylated PRL, as mimicked by the administration of S179D PRL, promotes differentiation.

PRL has long been described as a hormone important for both growth and differentiation in the mammary gland, but its precise contribution to each of these processes has proved difficult to ascertain. In part, this is due to complications introduced into experimental protocols by the luteotropic action of PRL in rodent models (1) and in part, in our opinion, it is because PRL has been thought of as a single substance. In this study, we have used an experimental approach which maintains the normal progesterone levels of pregnancy to test the effects of increased unmodified PRL and phosphorylated PRL on mammary gland development. PRL is produced in a variety of posttranslationally modified forms. We have focused our attention on the individual biological roles of unmodified PRL and phosphorylated PRL because a) these two forms between them constitute 98-100% of secreted pituitary PRL in the rodent (2-4) which serves as our experimental model, b) they have been demonstrated to have rather distinct biological activities (5-10), c) the proportion of each released from the pituitary is physiologically regulated (2,3), and d) phosphorylated PRL has been found in all species thus far examined (reviewed in 11). Standard preparations of PRL, such as those distributed by the NIDDK, contain a mixture of unmodified and phosphorylated PRL (5,6). Any biological activity observed as a result of treatment with these preparations therefore represents an aggregate activity related to the relative proportions of the unmodified and phosphorylated PRL present (5,6).

In order to determine the individual activities of unmodified and phosphorylated PRL in the mammary gland, we have administered recombinant versions of each to pregnant and non-pregnant animals, thereby altering the normal ratio of the different PRL forms in the animal. In the case of phosphorylated PRL, we have produced a molecular mimic, by substituting an aspartate residue for the normally phosphorylated serine, thereby producing S179D PRL (12,13). Aspartate mimicry of serine phosphorylation is used extensively in studies of enzymes activated or deactivated by phosphorylation and in several instances extensive structural analyses have confirmed complete three-dimensional as well as functional mimicry (e.g. 14,15). The recombinant wildtype hormone is identical to unmodified PRL with the exception, like S179D PRL, of an N-terminal extra methionine (13). The S179D PRL very effectively mimics the naturally phosphorylated molecule by acting, like phosphorylated PRL, as an extremely effective antagonist to U-PRL-induced Nb2 cell proliferation (5,13). The most important reason to use the molecular mimic rather than the naturally phosphorylated molecule is in order to prevent the possible interconversion of phosphorylated PRL to U-PRL by body phosphatases. Even though this is a very slow process (5,6), conversion would severely complicate interpretation of results.

We report very different activities of the two forms of PRL in the mammary gland which are independent of any luteotropic effect. Thus U-PRL promotes ductal and lobuloalveolar growth and S179D PRL inhibits growth and promotes formation of alveoli and β casein gene expression.

MATERIALS & METHODS

Animal experiments

Thirty-five, 16-week-old virgin female Sprague-Dawley rats were divided into 4 groups. Five rats served as non-pregnant controls, 10 rats as normal pregnant controls, 10 rats as recipients of unmodified PRL (U-PRL) and 10 rats as recipients of S179D PRL. Alzet minipumps (Alza, Palo Alto, CA) delivering 6 µg PRL/24h/rat were implanted subcutaneously the morning after vaginal plug observation. This was considered day 0.5 of pregnancy. On day 6.5, blood was obtained from the tails and collected into heparinized tubes. At term, dams were separated from their pups for 20h prior to sacrifice to standardize the histological appearance of the glands in each group. After sacrifice, the inguinal mammary glands were dissected out, measured and processed for whole mount or histological examination. Size was calculated by multiplying the length by the average width by the average depth. In a duplicate experiment, blood samples were taken from the tails on days 6.5 and 11.5 and from the trunk at day 19.5. No animal was bled more than once from the tail in order to keep stresses during pregnancy to a minimum. At the time of death (day 19.5 of pregnancy or day 21.5 shortly after pup delivery), inguinal mammary glands were snap frozen in liquid N₂ for later RNA extraction.

In a third and fourth experiment, non-pregnant females were treated with 24 µg of the PRLs/24h/rat for 4 days. At the time of sacrifice, trunk blood was collected and the inguinal mammary glands were again processed either for whole mount or regular histology.

All animal procedures were approved by the University of California, Riverside campus Committee on Laboratory Animal Care and were in accord with NIH guidelines.

Recombinant PRLs

Both recombinant human U-PRL and S179D PRL were produced and characterized as previously described (13). Both proteins were expressed and purified in parallel and were expressed at similar levels (13). The preparations were then tested for their activity in an Nb2 bioassay. U-PRL promotes Nb2 cell proliferation, while S179D PRL (like naturally phosphorylated PRL (5)) antagonizes this (13). The PRL preparations were concentrated to 1 mg/ml saline using Amicon Centripreps (Amicon, Danvers, MA) and loaded into model 2004 (first 2 experiments) or 2001 (third and fourth experiments) Alzet minipumps.

Histological analysis

Mammary glands were fixed in periodate-lysine-paraformaldehyde fixative (16) at 4 C overnight. The fixed tissue was dehydrated in a graded

ethanol series, cleared in Hemo De and then embedded in paraplast. Six micron sections were cut and stained with hematoxylin and eosin.

For morphometric analysis of the glands from non-pregnant animals, entire mammary glands were serially sectioned. Stained sections were viewed at a constant magnification using a PAXIT® (Midwest Information Systems, Franklin Park, IL) digital image system and the glands were measured on the screen. Every duct and associated dense stroma was measured on each section.

For whole mounts, glands were spread on glass and then fixed in Carnoy's solution (60% ethanol, 30% chloroform, 10% glacial acetic acid) for 60 min at room temperature. They were then washed in ethanol and defatted overnight in acetone. Defatted glands were then rehydrated and stained with carmine alum overnight at 4 C, dehydrated and cleared in methyl salicylate before mounting.

Hormone assays

All steroid hormones were measured by RIA using a kit from Diagnostic Products (Diagnostic Products Coat-a-Count, Los Angeles, CA). Progesterone levels were measured in serum (trunk blood) or plasma (tail blood). Previous work has demonstrated equivalent recognition of progesterone in rat serum and plasma with heparin as the anticoagulant (9). All results presented in a single table were produced in the same assay. Errors were therefore limited to intraassay variation. The coefficient of intraassay variation for this assay was 6.7% in our hands. Only trunk blood samples were assayed for corticosterone. As for progesterone, all results in the table were produced in the same assay. The coefficient of intraassay variation was 6.3% in our hands. Total estradiol levels were measured in trunk blood. The coefficient of intrassay variation was 5.7% in our hands.

Northern blot analysis for β casein gene expression

Total RNA was isolated from tissue or HC11 cells using the Trizol RNA reagent (Gibco BRL, NY). The isolated RNA was treated with DNase I (Gibco BRL, Gaithersburg, MD). Equal amounts of RNA (10 μ g) from control and test samples were loaded on a 1.0% agarose formaldehyde gel. The gels ran at 60 V for 3-5 h. The RNA was blotted onto nylon filters (Micron Separations, Inc., Westboro, MA) by capillary transfer with 10X SSC and fixed by UV cross-linking. The 201 bp probe used for hybridization was from a mouse β casein cDNA PCR product. The primers were: 5'-CCC GTC CCA CAA AAC ATC C-3' (forward); 5'-ATT AGC AAG ACT GGC AAG GCT G-3' (reverse). Probe was labeled with 50 μ Ci [α - 32 P] dCTP (ICN Biomedicals, Inc., Costa Mesa, CA) using a DECA Prime IITM DNA Labeling Kit (Ambion, Austin, TX). The labeled probes were separated by ProbeQuantTM G-50 Micro Columns (Amersham Pharmacia Biotech, Inc., Piscataway, NJ).

After 2 h of prehybridization at 65 C with the hybridization solution (25 M Na₂HPO₄, pH 7.2, 1 mM EDTA, pH 8.0, 7% SDS), hybridizations were carried out at 65 C for 16-24 h. The filters were then washed in alternating solutions of 20 mM Na₂HPO₄, pH 7.2, 1 mM EDTA, 5% SDS and 20 mM Na₂HPO₄, pH 7.2, 1 mM EDTA, 1% SDS for a total of 3 times in each. Filters were exposed to Fuji medical x-ray film (Fuji Medical Systems, Inc., Stamford, CT) for 1-7 days at -70 C.

Probe-stripping was performed by heating the nylon filter at 95 C for 10-30 min in a solution of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% SDS. A mouse 18S rRNA cDNA fragment (DECA templateTM-18S-mouse, 1212 bp) (Ambion, Austin, TX) was used to normalize for errors in RNA loading and transfer. A Kodak 1D Image Analysis System was used for quantification (Eastman Kodak Co, Rochester, NY).

Effect of U-PRL and S179D PRL on β -casein gene expression in HC11 cells

RPMI 1640 basal medium (Gibco BRL, Grand Island, NY), which contains 2 mM L-glutamine and 2 g/l NaHCO₃ served as a basal medium. HC11 cells were grown in RPMI 1640 growth medium containing 10% fetal calf serum (Gibco BRL, Grand Island, NY), 5 μ g/ml insulin (Sigma, St. Louis, MO), 10 ng/ml epidermal growth factor (Gibco BRL, Gaithersburg, MD), and 100 units/ml penicillin, and 100 μ g/ml streptomycin. Once HC11 cells became confluent, they were grown for 3 more days in growth medium. The medium was changed daily. On the third day post-confluency, the growth medium was removed and the cells washed 5 times with RPMI 1640 basal medium. The cells were refed with priming medium. Priming medium was RPMI 1640 basal medium supplemented with 10% charcoal-stripped horse serum (Cocalico Biologicals, Inc., Reamstown, PA), 100 units/ml penicillin, 100 μ g/ml streptomycin, 10 μ g/ml insulin and 1 μ g/ml hydrocortisone (Sigma, St. Louis, MO). The cells were kept in priming medium for 24h. The cells were then refed with induction medium. The

induction medium was priming medium to which 1 $\mu\text{g/ml}$ of the appropriate PRL was added. In the present study, cells were maintained in induction medium for 7 days and refed daily. The cells were collected for RNA isolation.

RESULTS

Pregnant animals

Gross observations

Treatment with U-PRL at 6 µg/24h/rat throughout pregnancy resulted in glands that were 1.5 fold the size of those from the untreated, pregnant animals ($102 \pm 7 \text{ mm}^3$, U-PRL; $67 \pm 5 \text{ mm}^3$, control). Treatment with S179D PRL at 6 µg/24h/rat throughout pregnancy, by contrast resulted in glands that were 40% smaller ($40.2 \pm 4.6 \text{ mm}^3$). As reported previously (8) the number of pup implantation sites per animal was indistinguishable among groups.

Histological observations

Figure 1 shows equivalent sections of mammary glands from each group of animals from the first experiment. Treatment with U-PRL resulted in larger lobuloalveoli than the control pregnant animals, while treatment with S179D PRL resulted in smaller lobuloalveoli than the controls. Even in the 40% smaller glands of the S179D PRL-treated animals, the area occupied by lobules was reduced. In other words, a greater amount of intervening connective tissue was evident. This resulted in lactational failure in previous experiments where this was monitored. A difference in morphological appearance due to milk composition was also evident in the different groups. Thus U-PRL treatment increased the number of lipid droplets, while S179D PRL treatment decreased the lipid content and increased the apparent protein content of the milk.

Hormone levels

Table 1 shows progesterone levels to be unaltered by U-PRL or S179D PRL treatment throughout pregnancy and estradiol and corticosterone levels to be unaltered on day 19.5 of pregnancy.

β casein expression

Figure 2 shows the result of Northern analysis for β casein expression in the day 19.5 and 21.5 samples. Combination of these two time points allowed for sufficient samples for good statistical analysis. U-PRL treatment caused reduced β casein gene expression while S179D PRL treatment caused an enhancement, the latter result consistent with the morphology of the glands showing an increased protein content. Because treatment with the PRLs was over and above the animal's own lactogens and normalization to ribosomal RNA can be questioned in glands with different epithelial to connective tissue ratios, we also examined the effects of the two PRLs on β casein gene expression in the HC11 mammary cell line. Figure 3 shows very clearly that S179D PRL is many fold more effective at inducing β casein gene expression than U-PRL in the 7 day treatment period.

Non-pregnant animals

By performing similar experiments in non-pregnant animals, it is possible to test whether the effects observed on the pregnant mammary gland were secondary to effects on placental lactogens. In addition, we could ask whether pregnancy levels of progesterone were required to observe these effects. Figure 4 shows the histology of mammary glands from animals treated with the PRLs at 24 $\mu\text{g}/24\text{h}/\text{rat}$ for 4 days. Both PRLs caused mammary development by comparison to the controls. However, U-PRL did this by promoting ductal growth and the growth of the relatively small number of lobules already present. S179D PRL, by contrast markedly increased the number of alveoli arising from smaller ducts. The overall picture is best illustrated by the whole mount images shown in Figure 5. Morphometric analysis of serial sections showed the ducts of the U-PRL treated glands to be 1.4 fold the diameter of the ducts in the S179D PRL treated glands (Table 2). The width of the associated dense stroma was, however, the same in both groups. Thus effects on ductal growth do not seem to be secondary to proliferation of the associated dense stroma.

Table 3 shows no statistically significant effect of the two PRLs on corticosterone or progesterone levels in these non-pregnant animals. Trends were, if anything, towards equally reduced corticosterone and progesterone with each PRL, i.e., towards an equal reduction in these other mammatropic hormones with both treatments.

DISCUSSION

Administration of additional U-PRL had very different effects on mammary gland histology from administration of S179D PRL. We can conclude therefore that the effects are not due to a simple elevation in total PRL, but are in fact specific to each form. Since there was no effect on progesterone, estradiol or corticosterone, we can conclude that the effects observed are not secondary to changes in these other mammatropic hormones. Similar effects in pregnant and non-pregnant animals demonstrates that these effects are not secondary to changes in placental lactogen. This was considered as a possibility because other investigators have a) demonstrated that mammary gland development was related to the number of developing pups (17), and b) because the levels of placental lactogens are very much in excess of PRL in the later stages of pregnancy (18) and placental lactogens are thought to function via the PRL receptor (19). Similar effects in pregnant and non-pregnant animals also tells us that pregnancy levels of progesterone are not required, although the current experiments do not address the question of the necessity for some progesterone or promotion of the processes by progesterone. Progesterone has been shown to upregulate PRL receptors on rodent mammary epithelium (20) and hence is likely to make the system more responsive to PRL in addition to having totally independent effects.

U-PRL significantly promotes ductal growth (as reflected in the diameter of ducts) in only 4 days at 24 $\mu\text{g}/24\text{h}$ in non-pregnant animals. This rate of administration results in circulating levels of 200 ng/ml by day 4, although days

1-3 have lower amounts as the PRL from the mini pump slowly equilibrates with tissue and blood compartments (9). At only 6 $\mu\text{g}/24\text{h}$, or 50 ng/ml (9), administered U-PRL results in a 50% increase in the overall size of the mammary gland at term. Some of this size increase was due to growth of lobuloalveoli, but some has to be the result of ductal growth in accord with the findings in non-pregnant animals. Since the gland as a whole was still contained within the fat pad, some general (as opposed to duct-associated dense) stromal proliferation is likely to have occurred. A similar concentration of circulating S179D PRL reduced the size of the mammary gland at term, i.e., it inhibited ductal growth and branching. At least part of this inhibition is likely due to the premature development of alveoli. Alveoli can be seen developing almost directly from large ducts in Figure 4D and E, i.e., they appear to cap branch points and prevent their further development. At the same time, S179D PRL promotes β casein gene expression. However, because the gland is too small, insufficient milk is produced to feed the pups.

When viewing the β casein expression data from the pregnancy experiment, it is important to remember that the effect is caused by administration of the recombinant PRLs over and above the rat's own PRL, which is a mixture of unmodified PRL and phosphorylated PRL. Changing the ratio by increasing U-PRL decreased β casein gene expression because it reduced the relative amount of phosphorylated PRL which is a much better stimulator of β casein expression. Thus the pregnancy data are concordant with the effects of the individual PRLs on the HC11 cells.

From these results it appears that U-PRL promotes overall growth of the mammary gland while S179D PRL, and presumably therefore phosphorylated PRL, promotes differentiation. Until now the effects of PRL on growth versus differentiation in the mammary gland were thought to be due to a change in the steroidal environment between pregnancy and lactation. This certainly plays a major role, but it is also clear that the forms of PRL are important. In regard to the forms of PRL, we have previously shown an increase in the ratio of U-PRL to phosphorylated PRL to occur during the latter two thirds of rodent pregnancy when the mammary gland is growing (3). Just before parturition, there is a peak of PRL (21) which is high in phosphorylated PRL (unpublished data) and phosphorylated PRL is very high in colostrum and milk (22,23) and the majority of PRL receptors are on the milk face of the mammary epithelium (24). Thus the ontogeny of PRL forms during pregnancy and lactation is concordant with the observed effects of the individual PRL forms on the mammary gland; first mostly growth and then mostly differentiation.

Recent work utilizing a variety of mammary epithelial and stromal transplant recombinations from the PRL receptor knockout mouse and the progesterone receptor knockout mouse supports our findings of a role for a lactogen in both ductal and alveolar growth, although the ductal growth was deduced in these studies to be indirect via effects on progesterone (25). While the

transplant studies show that progesterone plays a very important role in ductal growth, the transplant studies were qualitative and not quantitative and could easily have missed the additional contribution of PRL itself.

Our results showing effects of PRL on ductal growth without effects on progesterone levels suggest a direct effect of PRL on the duct. This is very much in keeping with the presence of PRL receptors in ductal epithelium (26). Alternatively or additionally, PRL may act indirectly via the stroma, although it is clear that the amount of dense stroma is not increased. Other investigators have implicated epidermal growth factor (27), transforming growth factor β (28), hepatocyte growth factor (29), insulin-like growth factor 1 (30) and vascular endothelial growth factor (31) as stromal factors which positively influence ductal growth. Rodent stroma, however, has been reported to be devoid of PRL receptors that would be required to effect such an indirect stimulation (26,32,33).

The different effects of the two forms of PRL in the mammary gland are probably the result of different signaling. These two forms of PRL have been shown to initiate different signaling cascades in Nb2 cells (10). Studies are ongoing to examine this issue in mammary cells.

In conclusion, we have clearly shown individual effects of the two forms of PRL on the mammary gland. U-PRL promotes growth while S179D PRL (pseudophosphorylated PRL) promotes differentiation. It is likely important that both forms are present throughout development of the mammary gland. Excesses of U-PRL might otherwise result in uncontrolled growth, while excesses of phosphorylated PRL would inhibit necessary growth and cell replacement. The proportion of U-PRL to phosphorylated PRL must change during development of the mammary gland in preparation for lactation such that growth initially predominates and is later superseded by differentiated function. A substance such as S179D PRL which inhibits growth and promotes differentiation may have potential in the treatment or prevention of breast cancer.

REFERENCES

1. **Hsueh AJ, Adashi EY, Jones PB, Welsh TH Jr.** Hormonal regulation of the differentiation of cultured ovarian granulosa cells. *Endocrine Reviews* 5: 76-127, 1984.
2. **Ho TWC, Leong FS, Olaso CH, Walker AM.** Secretion of specific non-phosphorylated and phosphorylated rat prolactin isoforms at different stages of the estrous cycle. *Neuroendocrinology* 58: 160-165, 1993.
3. **Ho TWC, Kawaminami M, Walker AM.** Secretion of phosphorylated and non-phosphorylated monomer prolactin isoforms during rat pregnancy and pseudopregnancy. *Endocrine J* (now *Endocrine*) 1: 435-439, 1993.
4. **Oetting WS, Walker AM.** Differential isoform distribution between stored and secreted prolactin. *Endocrinology* 119: 1377-1381, 1986.
5. **Wang Y-F, Walker AM.** Dephosphorylation of standard prolactin produces a more biologically active molecule. Evidence for antagonism between non-phosphorylated and phosphorylated prolactin in the stimulation of Nb2 cell proliferation. *Endocrinology* 133: 2156-2160, 1993.
6. **Krown KA, Wang Y-F, Ho TWC, Kelly PA, Walker AM.** Prolactin isoform 2 as an autocrine growth factor for GH3 cells. *Endocrinology* 131: 595-602, 1992.
7. **Ho TWC, Greenan JR, Walker AM.** Mammoth autoregulation: The differential roles of the isoforms of prolactin. *Endocrinology* 124: 1507-1514, 1989.
8. **Yang L, Kuo CB, Liu Y, Coss D, Xu XL, Chen C, Oster-Granite ML, Walker AM.** Administration of unmodified prolactin (U-PRL) and a molecular mimic of phosphorylated prolactin (PP-PRL) during rat pregnancy provides evidence that the U-PRL:PP-PRL ratio is crucial to the normal development of pup tissues. *J Endocrinol* 168: 227-238, 2001.
9. **Coss DJ, Yang L, Kuo CB, Xu X, Luben RA, Walker AM.** Effects of prolactin on osteoblast alkaline phosphatase and bone formation in the developing rat. *Am J Physiol* 279: E1216-E1225, 2000.
10. **Coss D, Kuo C-YB, Yang L, Ingleton P, Luben R, Walker AM.** Dissociation of Janus kinase 2 and signal transducer and activator of transcription 5 activation after treatment of Nb2 cells with a molecular mimic of phosphorylated prolactin. *Endocrinology* 140: 5087-5094, 1999.

11. **Lorenson MY, Walker AM.** Structure-function relationships in prolactin. In *Prolactin*, Horseman, N.D., ed., Kluwer Academic Publishers, Norwell, MA, pp. 189-217, 2001
12. **Wang Y-F, Liu J-W, Mamidi M, Walker AM.** Identification of the major site of rat prolactin phosphorylation as serine 177. *J Biol Chem* 271: 2462-2469, 1996.
13. **Chen TJ, Kuo C-YB, Tsai KF, Liu J-W, Chen D-Y, Walker AM.** Development of recombinant human prolactin receptor antagonists by molecular mimicry of the phosphorylated hormone. *Endocrinology* 139: 609-616, 1998.
14. **Thorsness PE, Koshland Jr DE.** Inactivation of isocitrate dehydrogenase by phosphorylation is mediated by the negative charge of the phosphate. *J Biol Chem* 262: 10422-10425, 1987.
15. **Wittekind M, Reizer J, Deutscher J, Saier MH, Klevit RE.** Common structural changes accompany the functional inactivation of HPr by seryl phosphorylation or by serine to aspartate substitution. *Biochemistry* 28: 9908-9912, 1989.
16. **McLean IW, Nakane PK.** Periodate-lysine-paraformaldehyde fixative. A new fixation for immunoelectron microscopy. *J Histochem Cytochem* 22: 1077-1083, 1974.
17. **Nagasawa H, Yanai R.** Quantitative participation of placental mammatropic hormones in mammary development during pregnancy of mice. *Endocrinol Jpn* 18: 507-510, 1971.
18. **Robertson MC, Friesen HC.** Two forms of rat placental lactogen revealed by radioimmunoassay. *Endocrinology* 108: 2388-2390, 1981.
19. **Freemark M, Kirk K, Pihoker C, Robertson MC, Shiu RPC, Driscoll P.** Pregnancy lactogens in the rat conceptus and fetus: circulating levels, distribution of binding, and expression of receptor messenger ribonucleic acid. *Endocrinology* 133: 1830-1842, 1993.
20. **Edery M, Imagawa W, Larson L, Nandi S.** Regulation of estrogen and progesterone receptor levels in mouse mammary epithelial cells grown in serum-free collagen gel culture. *Endocrinology* 116: 105-112, 1985.
21. **Fliestra RJ, Voogt JL.** Lactogenic hormone of the placenta and pituitary inhibit suckling-induced prolactin (PRL) release but not the antepartum PRL surge. *Proc Soc Exp Biol Med* 214: 258-264, 1997.

22. **Kacsoh B, Veress Z, Toth BE, Avery LM, Grosvenor CE.** Bioactive and immunoreactive variants of prolactin in milk and serum of lactating rats and their pups. *J Endocrinol* 138: 243-257, 1993.
23. **Ellis L, Picciano MF** Prolactin variants in term and preterm human milk: altered structural characteristics, biological activity and immunoreactivity. *Endocrine Regulations* 27: 181-192, 1993.
24. **Clevenger CV, Chang W-P, Ngo W, Pasha TLM, Montone K, Tomaszewski JE.** Expression of prolactin and prolactin receptor in human breast carcinoma. Evidence for an autocrine/paracrine loop. *Am J Pathol* 146: 695-705, 1995.
25. **Ormandy CJ, Horseman ND, Naylor MJ, Harris J, Robertson F, Binart N, Kelly PA.** Mammary gland development. In: *Prolactin*. Horseman, ND, ed. Kluwer Academic Publishers, Norwell, MA, 2001, p. 219-232.
26. **Ouhtit A, Morel G, Kelly PA.** Visualization of gene expression of short and long forms of prolactin receptor in rat reproductive tissues. *Biol Reprod* 49: 528-536, 1993.
27. **Wiesen JF, Young P, Werb Z, Cunha GR.** Signaling through the stromal epidermal growth factor receptor is necessary for mammary ductal development. *Development* 126: 335-344, 1999.
28. **Daniel CW, Robinson S, Silberstein GB.** The role of TGF-beta in patterning and growth of the mammary ductal tree. *J Mamm Gland Biol Neoplasia* 1: 331-341, 1996.
29. **Soriano JV, Pepper MS, Orci L, Montesano R.** Roles of hepatocyte growth factor/scatter factor and transforming growth factor-beta 1 in mammary gland ductal morphogenesis. *J Mamm Gland Biol Neoplasia* 3: 133-150, 1998.
30. **Kleinberg DL, Feldman M, Ruan M.** IGF-I: an essential factor in terminal end bud formation and ductal morphogenesis. *J Mamm Gland Biol Neoplasia* 5: 7-17, 2000.
31. **Pepper MS, Baetens D, Mandriota SJ, Di Sanza C, Oikemus S, Lane TF, Soriano JV, Montesano R, Iruela-Arispe ML.** Regulation of VEGF and VEGF receptor expression in the rodent mammary gland during pregnancy, lactation and involution. *Dev Dynamics* 218: 507-524, 2000.
32. **Ouhtit A, Morel G, Kelly PA.** Visualization of gene expression of short and long forms of prolactin receptor in the rat. *Endocrinology* 133: 135-144, 1993.

33. **Meister B, Jacobsson G, Elde R.** Observations on the localization of prolactin receptor mRNA in rat tissues as revealed by in situ hybridization. *Acta Physiol Scand* 146: 533-534, 1992.

ACKNOWLEDGEMENTS

The authors would like to thank Jeffrey Rosen (Baylor University, Houston, TX) for provision of mouse β casein cDNA, Margaret Neville (University of Colorado, Denver, CO) and Nancy Hynes (Friedrich Miescher Inst., Basel, Switzerland) for provision of the HC11 cells, Sally Scott for expert animal husbandry, Hsiao-li Wu for preparation of the illustrations and Nancy Price for preparation of the manuscript.

	Day	Control	U-PRL	S179D PRL
Progesterone (ng/ml)	6.5	374 ± 37	351 ± 20	323 ± 28
	11.5	369 ± 20	380 ± 20	389 ± 43
	19.5	377 ± 23	283 ± 28	328 ± 18
Estradiol (pg/ml)	19.5	15 ± 1.3	14.3 ± 1	15.1 ± 2
Corticosterone (ng/ml)	19.5	138 ± 29	205 ± 62	169 ± 22

Table 1: Levels of mammatropic steroids during pregnancy in the three treatment groups.

	Duct	Stroma	Ratio (S:D)
U-PRL	11.18 ± 1.4*	22.12 ± 1.56	1.98
S179D PRL	8.5 ± 1.3*	23.33 ± 1.95	2.7

Table 2. Duct diameters and associated stroma widths in cross sections in the 4 day treated non-pregnant animals. Numbers are in relative units. * indicates statistically significantly different with $p < 0.05$. All cross sectional views on 54 sections per gland were analyzed.

	Control	U-PRL	S179D PRL
Progesterone (ng/ml)	25.2 ± 8	14.6 ± 5.3	17.4 ± 4.4
Corticosterone (ng/ml)	331 ± 85	280 ± 32	256 ± 66

Table 3: Levels of progesterone and corticosterone in the 4-5 day treated non-pregnant animals.

FIGURE LEGENDS

Figure 1. Term mammary glands from each treatment group. A, control rats; B, rats treated with U-PRL; C, rats treated with S179D PRL.

Figure 2. Northern analysis of β casein mRNA expression in mammary glands at days 19.5 and at term. ($n = 8$ rats per group). CON, control rats receiving no additional PRL. Differences among groups were analyzed by ANOVA and individual posttests. * indicates significantly different from the control group. $p < 0.05$.

Figure 3. Northern analysis of β casein mRNA expression in HC11 cells in response to the different PRLs. Differences among groups were analyzed by ANOVA with posttests. *, $p < 0.001$ versus control (CON) and U-PRL. $n =$ five separate experiments.

Figure 4. Non-pregnant mammary glands treated with the different PRLs for 4 days. A, control rats; B, rats treated with U-PRL, C, rats treated with S179D PRL; D & E, higher magnification views of mammary glands from rats treated with S179D PRL. Magnification A-C, 100X; D, E, 200X.

Figure 5. Whole mount glands from non-pregnant animals treated for 5 days with the different PRLs. A, treatment with U-PRL; B, treatment with S179D PRL. Note the fewer, but larger alveoli in panel A and the multiple smaller alveoli in panel B.

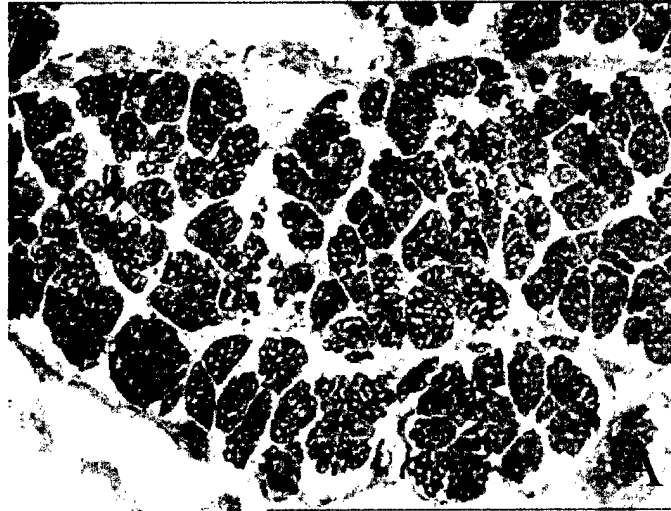


Figure 1

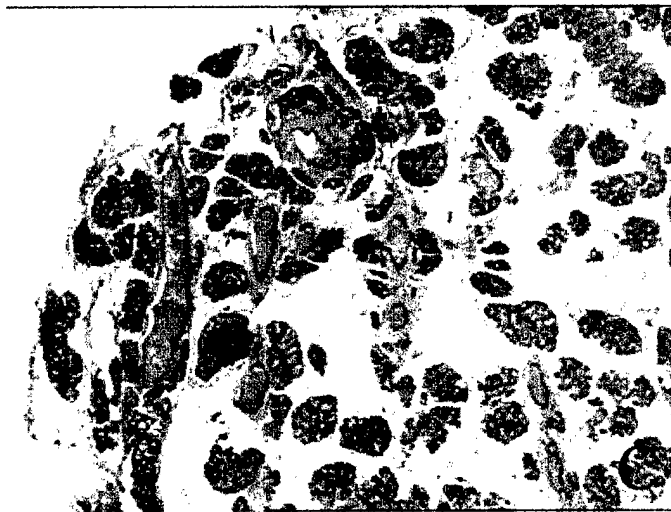
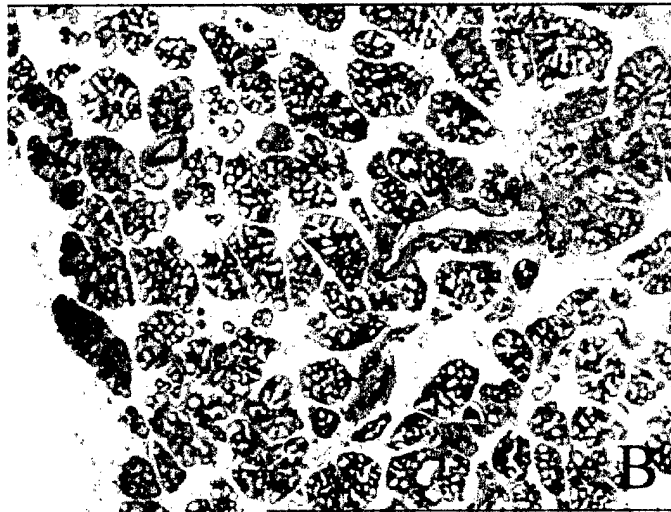


Figure 2

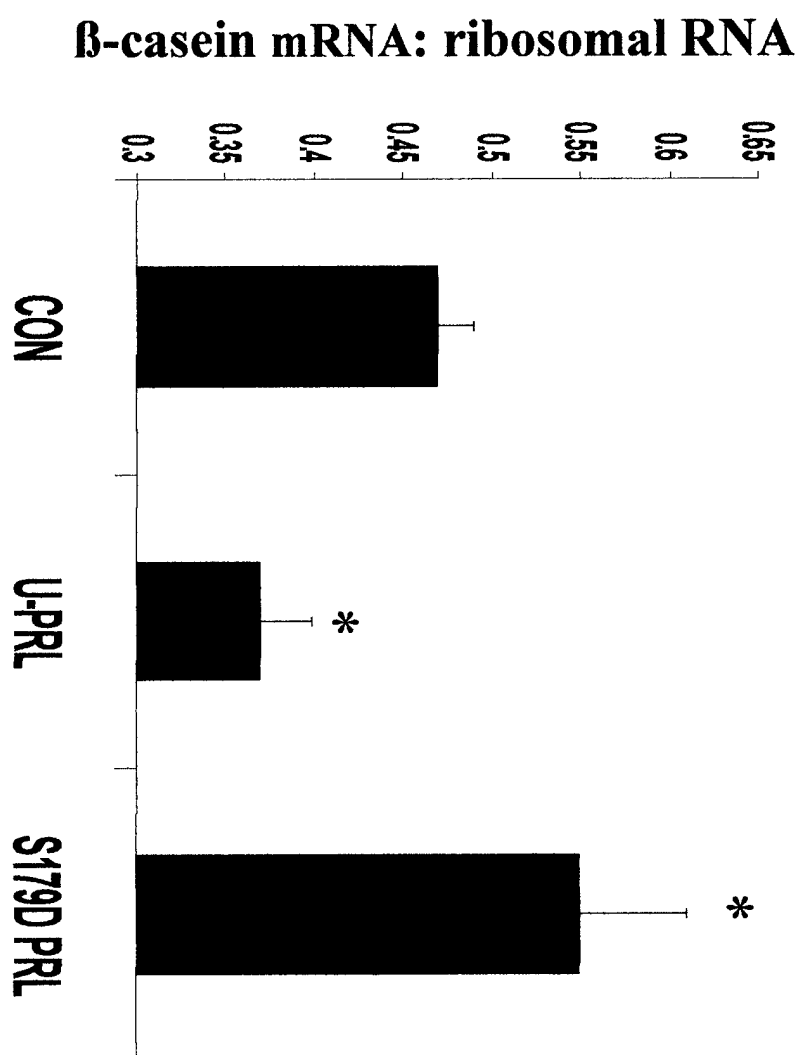
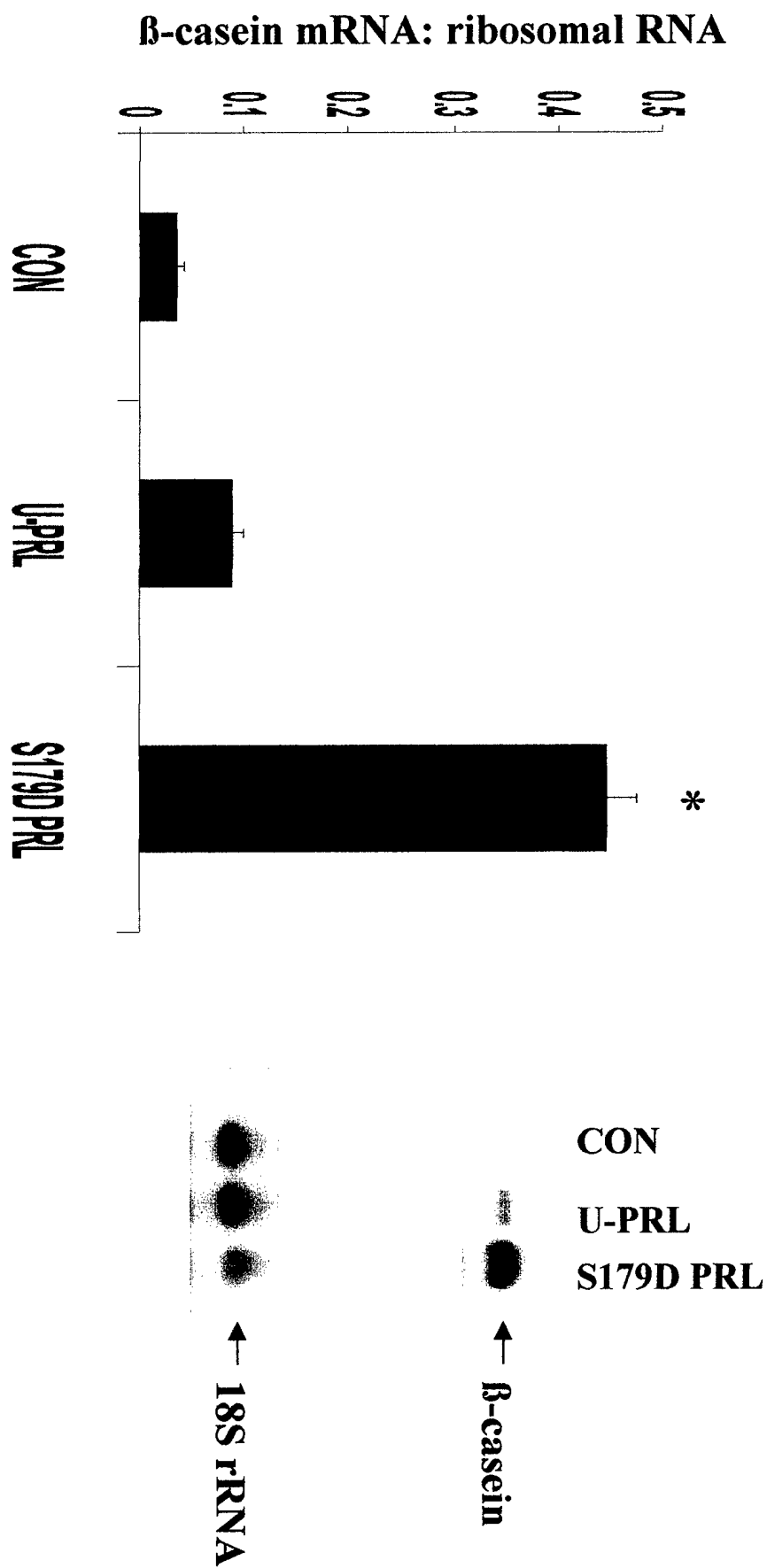


Figure 3



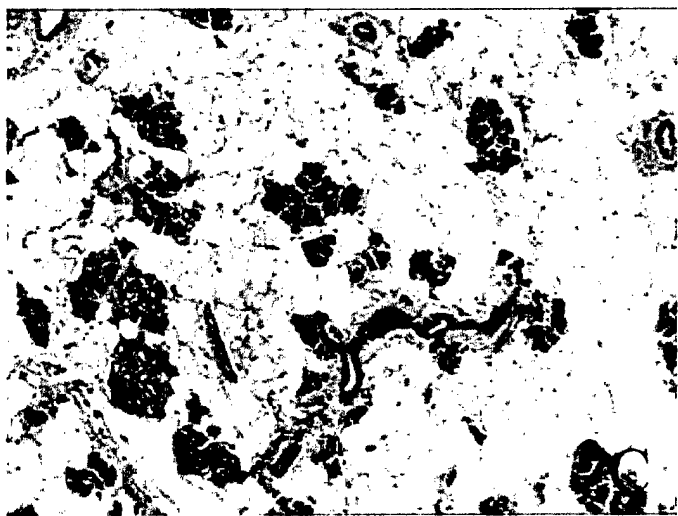
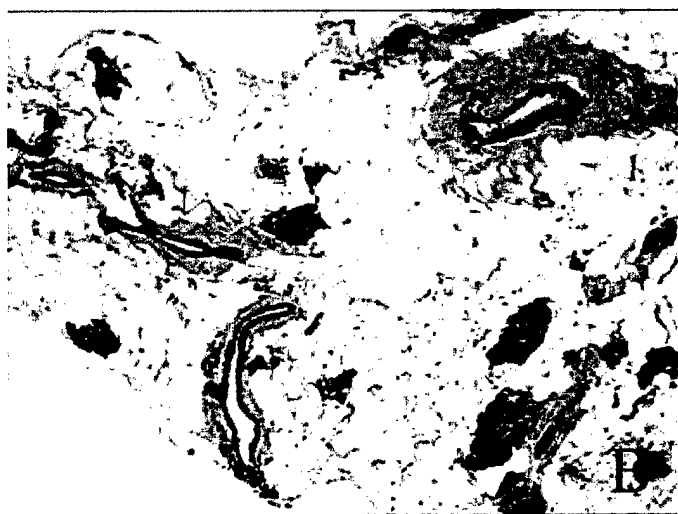
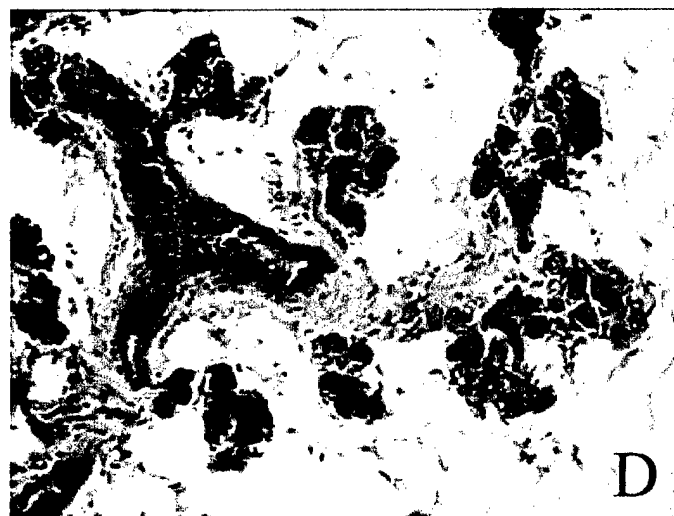
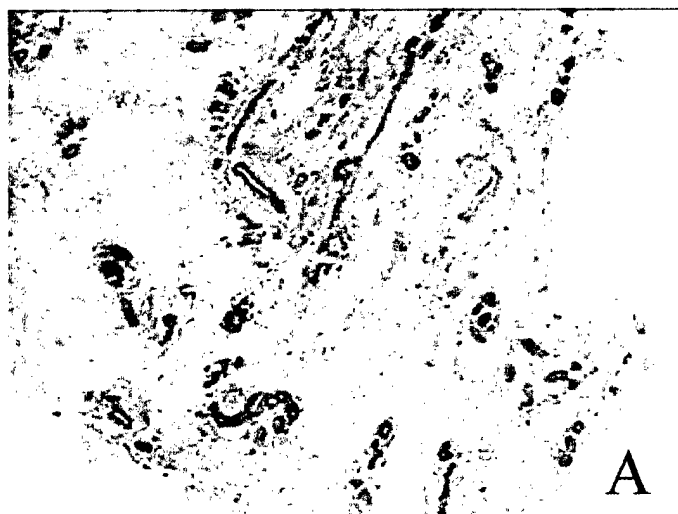
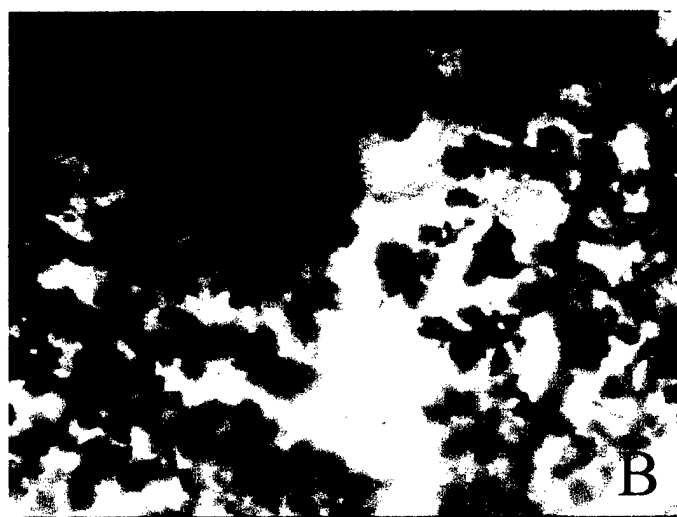
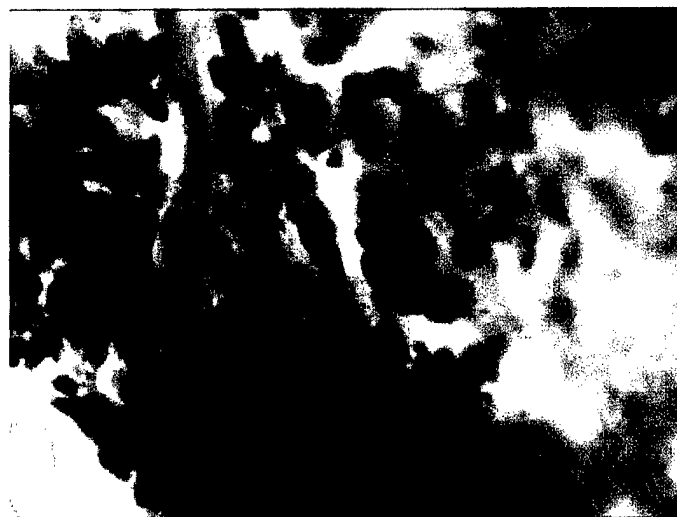


Figure 4

Figure 5



Abstract Preview

SIGNALING AND BIOLOGICAL ACTIVITY OF A MOLECULAR MIMIC OF PHOSPHORYLATED PROLACTIN.

A.M. Walker. ¹Biomedical Sciences, University of California, Riverside, CA, United States

PRL has been described as important for both growth and differentiation in the mammary gland, but its precise contribution to each of these processes has proved difficult to ascertain. In part, this is due to complications introduced into experimental protocols by the luteotropic action of PRL in rodent models and in part, in our opinion, because PRL has been thought of as a single substance. PRL has been shown to be posttranslationally modified in several ways. In rodents, a single posttranslational modification predominates. Thus unmodified PRL (U-PRL) and phosphorylated PRL (P-PRL) together make up 98-100% of the PRL released from the pituitary. In order to study the specific roles of U- and P-PRL, we have produced recombinant versions of each. Recombinant P-PRL was produced by molecular mimicry--substituting an aspartate for the normally phosphorylated serine, thereby producing S179D PRL. Physiological analyses have shown that the ratio of U- to P-PRL changes with time during pregnancy and lactation. Because both PRL forms are always present, we have studied their roles *in vivo* by adjusting the relative ratio of each form in the animal. Administration of either PRL to pregnant and non-pregnant animals was without effect on the levels of progesterone (P), estrogen and corticosterone. Additional U-PRL during pregnancy stimulated ductal and alveolar growth. Additional S179D PRL inhibited ductal and alveolar growth while promoting the formation of alveoli and β -casein gene expression. These effects required neither the levels of P normal to pregnancy nor placental products since they were duplicated in non-pregnant animals. Elevated P, however, increased the growth-promoting effects of U-PRL in the ducts. Thus it appears that the effects of U-PRL tend towards growth in the mammary gland while those of P-PRL tend towards differentiation. To examine this in more detail, we have assessed the individual roles of the two PRLs on signal transduction in HC11 cells. Compared with U-PRL, S179D PRL resulted in decreased tyrosine, and increased serine, phosphorylation of STAT 5a, increased β -casein gene expression and increased expression of the short PRL receptor suggesting that differentiative effects of P-PRL may be mediated in part through interaction with the short form of the PRL receptor.

Abstract Central © 1.3

Copyright © ScholarOne, Inc., 2000. All Rights Reserved.

[Terms and Conditions of Use](#)

Abstract Preview

DIFFERENTIAL MODULATION OF THE EXPRESSION OF THE LONG AND SHORT FORM OF THE PRL RECEPTOR AND -CASEIN BY UNMODIFIED PRL AND A MOLECULAR MIMIC OF PHOSPHORYLATED PRL SUGGESTS THAT THE SHORT FORM OF THE RECEPTOR DOES NOT ACT AS A DOMINANT NEGATIVE FOR SIGNALLING RESULTING IN -CASEIN EXPRESSION.

W. Wu¹, X. Xu¹ and A.M. Walker¹. ¹Biomedical Sciences, University of California, Riverside, CA, United States

HCII mouse mammary cells were grown in RPMI 1640 containing 10% FBS, 5 µg/ml insulin and 10 ng/ml epidermal growth factor (EGF). Once confluent, the medium was changed daily. On the third day post-confluency, the growth medium was removed, the cells washed 5 times to remove EGF and then incubated in RPMI 1640 supplemented with 10% charcoal-stripped horse serum, 10 µg/ml insulin and 1 µg/ml hydrocortisone for 24h (priming medium). At the end of the 24h period, the priming medium was changed and then further supplemented with 0 or 1 µg/ml unmodified recombinant human PRL (WT PRL) or the molecular mimic of human phosphorylated PRL (S179D PRL). Exposure to the PRLs continued for 7 days, during which time the medium was changed daily. Total RNA was extracted, treated with DNase I, run on a 1% agarose formaldehyde gel at 10 µg/lane and then transferred to a nylon filter. Probes for Northern blots were produced from purified RT-PCR products of rat PRL (399 bp) and mouse β-casein (201 bp) mRNA. The probe for the PRL receptor recognized domains common to all forms. All image data were normalized for loading and transfer using a 1.2 kb cDNA of mouse 18S rRNA. Treatment with No PRL, WT PRL or S179D PRL resulted in different ratios of short (S) to long (L) receptor and different degrees of β-casein gene expression. Thus an S:L ratio of 5.2 in the cells not exposed to PRL resulted in a β-casein expression level designated as 1. Treatment with WT PRL elevated the S:L (by decreasing L) to 6.04 and β-casein expression to 10.8. Treatment with S179D PRL elevated the S:L (by decreasing L and elevating S) to 6.96 and the β-casein to 19.23. Thus a linear relationship was obtained whereby β-casein expression was a direct function of S:L. This result argues against the idea that S functions as a dominant negative to β-casein gene expression and in fact supports the idea that an increase in S:L expression is directly responsible for β-casein upregulation. USARMC #BC 990711

Abstract Central © 1.3

Copyright © ScholarOne, Inc., 2000. All Rights Reserved.

[Terms and Conditions of Use](#)